



Avvisati, R., Meringolo, M., Stendardo, E., Malavasi, E., Marinelli, S., & Badiani, A. (2018). Intravenous self-administration of benzydamine, a non-steroidal anti-inflammatory drug with a central cannabinoidergic mechanism of action. *Addiction Biology*, 23(2), 610-619.  
<https://doi.org/10.1111/adb.12516>

Peer reviewed version

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[10.1111/adb.12516](https://doi.org/10.1111/adb.12516)

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**Intravenous self-administration of benzydamine, a non-steroidal anti-inflammatory drug with a central cannabinoidergic mechanism of action.**

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A revised version of this manuscript has been accepted for publication in *Addiction Biology* and is available at <https://onlinelibrary.wiley.com/doi/full/10.1111/adb.12516>.

## ABSTRACT

Benzydamine (BZY) is a non-steroidal anti-inflammatory drug (NSAID) used for the topical treatment of inflammations of the oral and vaginal mucosa. Virtually nothing is known about the central pharmacological actions of BZY. Yet, there are reports of voluntary systemic overdosage of BZY in drug addicts, resulting in a euphoric, hallucinatory state. In the present study, we investigated the reward properties of BZY in a rat self-administration paradigm. We found that BZY has a powerful reinforcing effect and that this effect is greatly facilitated in animals that already had substance experience, having previously self-administered heroin and cocaine, indicating cross sensitization between BZY and other common drugs of abuse. We then assessed the effect of BZY on Prelimbic Cortex-to-Nucleus Accumbens (PLCx-NAcc) glutamatergic transmission, using field recordings in rat parasagittal brain slices. BZY dose-dependently reduced both field excitatory post synaptic potential (fEPSP) amplitude and paired pulse ratio, suggesting a presynaptic mechanism of action. Similarly to the *in vivo* paradigm, also the electrophysiological effects of BZY were potentiated in slices from animals that had undergone cocaine and heroin self-administration. Furthermore, BZY-induced LTD-like responses in the PLCx-NAcc circuitry, were significantly reduced in the presence of the CB1 receptor antagonist AM251. These findings provide firm evidence of the abuse liability of BZY and suggest a possible cannabinoidergic mechanism of action. Further research is needed in order to give insights into the molecular mechanism underlying BZY psychoactive and reinforcing effects, to better understand its abuse potential and possibly redefine the toxicological profile of this drug.

**Keywords:** Benzydamine, cannabinoid receptor type 1, drug abuse, fEPSP, NSAID, self-administration

## INTRODUCTION

Benzydamine (BZY) is a non-steroidal anti-inflammatory drug that was synthesized by Angelini's laboratories in 1964 and made commercially available since 1966 for the symptomatic treatment of acute inflammatory states of the oral and vaginal mucosae. A number of pharmaceutical preparations for topical use are available (e.g., TANTUM® Verde and GineTANTUM® in Italy, Difflam® in the UK, Tanflex® and Benzidan® in Turkey, Flogo-Rosa® in Brazil). Benzydamine is also contained in medications for systemic use (with concentrations of BZY ten times lower than those for topical use), as in the case of Benflogin® in Brazil, which, however, is no longer available on the market.

The mechanisms of action of BZY are complex. The anti-inflammatory effect mainly depends on the inhibition of prostaglandin synthesis, probably due to disruption of calcium mobilization/sequestration at the level the plasma membrane of inflammatory cells (Jeremy et al., 1991). Benzydamine also exhibits a less specific mechanism of action termed 'membrane stabilization', resulting in the inhibition of granule release by neutrophils and lysosomes (Quane et al., 1998). In addition to the anti-inflammatory/analgesic effect, BZY has local anesthetic effects (Sato and Maehara, 1967; Silvestrini et al., 1966a, 1966b).

Relatively little is known about the effects of BZY on the central nervous system. Symptoms such as dizziness, hallucinations, and anxiety have been reported as a consequence of unintended systemic ingestion (Gómez-López et al., 1999; Ballesteros et al., 2009; Settimi et al., 2012; Acar et al., 2014). Furthermore, there is evidence of recreational use of BZY in various countries, including Brazil, Italy, Poland, and Turkey (Nappo et al., 1993; Anand et al., 2007; Opaleye et al., 2009, 2011; Settimi et al., 2012; Balaban et al., 2013; Barwina et al., 2014). In Brazil, for example, BZY has been popular among drug-

experienced street youth since at least the early 1990s, probably due to its low price and ready availability (Nappo et al., 1993). In the following two decades its use spread to Brazil club scene, with the blossoming of theme rave parties (Benflogin® parties) and pop songs titled 'Benzydamine'. Informal self-reports, hosted by internet drug forums and social networks, detail the psychotropic effects of BZY and provide information about route of administration, dosage, and substance preparation from commercial preparations, as well as advice about other psychotropic substances to be assumed with BZY to enhance its pleasurable effects and dampen the undesired ones (Souza et al., 2008). Indeed, BZY seems to be particularly popular among poly-drug users (Anand et al., 2007; Opaleye et al., 2009). We have recently reported BZY use among the outpatients of an addiction clinic (Villa Maraini in Rome, Italy) who co-abused heroin and cocaine (Malavasi et al., 2012).

In the present study, in order to better understand BZY abuse potential, we investigated the ability of this drug to sustain intravenous self-administration in drug-naïve rats. To verify whether pre-exposure to addictive drugs would facilitate the reinforcing effects of BZY, we also investigated BZY self-administration in rats that had been previously trained to self-administer cocaine and heroin (to mimic the drug history of polydrug abusers). Finally, we investigated the electrophysiological effects of BZY on the PLCx-NAcc synapses, which are thought to play an important role in drug reward and drug seeking (Ikemoto and Panksepp, 1999; Kalivas and Volkow, 2005; Kalivas et al., 2009). This circuitry has been shown to undergo neuroplasticity changes following repeated exposure to addictive drugs, which has been implicated in the development of drug addiction (Van den Oever et al., 2012; Quintero 2013; Kalivas et al., 2009).

## MATERIALS AND METHODS

### Experiment 1: BZY self-administration

*Animals and surgery:* All the experimental procedures were carried out according the guidelines established by the European legislation (Directive 2010/63/EU) and the Italian legislation (L.D. 26/2014).

A total of 23 male Sprague-Dawley rats (Harlan Laboratories) weighting 250-300 g upon arrival were used. The rats were housed in pairs in transparent plastic cages (40 cm in length, 24.5 cm in width, and 18 cm in height) with stainless steel grid tops and flat bottoms covered with ground corncob bedding, in a temperature ( $21\pm1$  °C) and humidity (70%) controlled room with a 14-h dark/10-h light cycle (lights off at 0700 hours). The rats were gently handled twice a week for two weeks before undergoing surgical catheterization, following procedures described previously in detail (Caprioli et al., 2007, 2008). On the day of surgery, the rats received an i.p. injection of 2.33 mg of xylazine hydrochloride (Rompun®, Bayer HealthCare) and 0.56 ml/kg of Zoletil 100® (Virbac, Carros, France), containing tiletamine (50 mg/ml) and zolazepam (50 mg/ml). The catheter consisted of 10.5 cm of silicone tubing (0.37-mm inner diameter and 0.94-mm outer diameter) sheathed at 3.4 cm from its proximal end by a 5-mm piece of heat-shrink tubing. The catheter was inserted into the right jugular vein and secured to the surrounding soft tissues with silk thread. Catheter distal end was externalized through a small incision at the nape of the neck and connected to an L-shaped 22-gauge cannula, which was secured to rat's skull using dental cement and stainless steel screws. After surgery, the rats were given 15 mg i.v. enrofloxacin (Baytril®, KVP Pharma + Veterinär Produkte GmbH, Kiel, Germany). The catheters were flushed daily (1800 hours) with 0.1 ml of sterile saline solution containing 0.4 mg of enrofloxacin and 25

IU heparin (Marvecs Services, Agrate Brianza, Italy). The rats had ad libitum access to food and water throughout the experiment, except during the self-administration sessions. The rats were allowed to recover from the surgery for 7-10 days and were then assigned to either the drug-naïve group (n=12) or the drug-experienced group (n=11) before the start of drug self-administration.

*Apparatus:* The apparatus consisted of self-administration cages (28.5-cm length, 27-cm width, and 32-cm height) made of transparent plastic (front and rear walls), aluminum (sidewalls and ceiling), and stainless steel (grid floor). Plastic trays covered with pinewood shaving were placed under the cage floors. Each cage was equipped with two retractable levers, positioned on the left-hand wall 12.5 cm apart and 9 cm above the floor, two white cue lights, positioned above each lever, and a counterbalanced arm holding a liquid swivel. Cages and accessories were purchased from ESATEL S.r.l. (Rome, Italy). The self-administration cages were placed within sound- and light-attenuating cubicles. Each cage was connected via an electronic interface to a syringe pump (Razel Scientific Instruments, St. Albans, VT, USA) and to a programmable logic controller (PLC; Allen Bradley, Milwaukee, WI, USA). Finally, the PLCs were connected to PCs running control software developed by Aries Sistemi S.r.l. (Rome, Italy).

*Self-administration procedures in the drug-naïve group:* The rats in the drug-naïve group underwent fourteen 3-h daily sessions of BZY self-administration. Independent groups (n=4) self-administered one of three infusion doses of BZY (Sigma Aldrich): 250, 500, and 1000 µg/kg (dissolved in 40 µl of sterile saline solution). The sessions took place during the dark phase between 0900 and 1700 hours. During the session both levers were extended, but only one of them (counterbalanced across animals) was 'active' whereas pressing on the



other lever had no scheduled consequences except resetting the counter for the active lever. The number of consecutive presses (on the active lever) required to obtain a single drug infusion (fixed ratio, FR) was progressively increased during training: FR1 on sessions 1-7, FR2 on session 8, FR3 on session 9, FR4 on session 10, and finally FR5 on sessions 11-14. Once the appropriate FR had been reached, a drug infusion was delivered over a period of 3 s and at the same time both levers retracted for a time-out period of 40 s. A white cue light above the active lever was on throughout the session except during the time-out period. Rats that did not spontaneously self-administer at least one infusion within the first 5 min of the session were placed with their forepaws on the active lever, so as to trigger a priming infusion. Priming infusions were administered again at times 60 and 120 min to animals that had not spontaneously self-administered at least one infusion during time periods 5-60 and 60-120 min. On FR5 sessions (days 11-14) priming infusions were given only to rats that failed to self-administer at least one infusion within the 0-5 min period. These experimenter-induced lever presses were opportunistically subtracted from the total. The rats were allowed to self-administer a maximum of 50 infusions within a single session.

*Self-administration procedures in the drug-experienced group:* The rats in the drug-experienced group were first trained to self-administer cocaine and heroin on alternate days for 14 consecutive sessions (following procedures previously described in detail by Caprioli et al., 2009 and Montanari et al., 2015) and then underwent 10 extinction sessions, during which lever pressing had no scheduled consequences. After completion of this procedure, they were moved in their home cages to a different experimental room and given a 5-day period of rest, after which the rats were trained to self-administer one of three infusion doses of BZY (n=3-4), as described above for drug-naïve rats.

## Experiment 2: Slice electrophysiology

*Animals:* Acute brain slices were obtained from 18 drug-naïve and 5 drug-experienced male Sprague-Dawley rats of the same age of those used in the BZY self-administration experiment (P60-90 for drug-naïve rats and P80-100 for drug-experienced rats). Drug-experienced animals underwent alternate cocaine and heroin self-administration training as detailed in the previous experiment and were then given 5-7 days of rest in their home cage before being anesthetized for brain slice collection. Notice that the rats used in the electrophysiology experiments did not receive BZY self-administration training.

*Slice collection:* Animals were deeply anesthetized with halothane (2-bromo-2-chloro-1,1,1-trifluoroethane, Sigma-Aldrich) and decapitated. The brain was rapidly removed and 300  $\mu\text{m}$  thick, parasagittal slices were obtained using a Leica 1200T vibratome, and immersed in a cutting solution containing (in mM): KCl 2.5,  $\text{NaH}_2\text{PO}_4$  1.25,  $\text{MgSO}_4$  10,  $\text{CaCl}_2$  0.5, Choline 120,  $\text{NaHCO}_3$  26, Glucose 10. The slices were then incubated in artificial cerebrospinal fluid (aCSF) in a holding chamber at 32°C for the first 60 min and at room temperature thereafter. Each slice was transferred to a recording chamber constantly perfused with aCSF (31-32°C, 3-4 ml/min) with the following composition (in mM): NaCl 126, KCl 1.25,  $\text{NaH}_2\text{PO}_4$  1.25,  $\text{MgSO}_4$  2,  $\text{CaCl}_2$  2,  $\text{NaHCO}_3$  26, Glucose 10. All solutions were saturated with a 95%  $\text{O}_2$  – 5%  $\text{CO}_2$  gas mixture.

*Stimulation and recordings:* Field excitatory post synaptic potentials (fEPSPs) were recorded by placing a glass recording electrode, filled with aCSF, in the Core of the Nucleus Accumbens (NAcc-Core). Prelimbic cortical afferents were stimulated by a 25  $\mu\text{s}$  square wave current delivered at 0.03 Hz (a sweep every 30 s) through a bipolar stainless steel microelectrode placed at the border between PLCx and NAcc. The GABA<sub>A</sub>R antagonist

picrotoxin (100  $\mu$ M) was added to the perfusion aCSF due to presence of strong GABAergic inhibition in the NAcc-Core. Signals were fed into a Multiclamp 700b amplifier (Axon Instruments), filtered at 1 kHz, converted by a Digidata 1322A (Axon Instruments), acquired and analyzed using Clampex 9.2 software.

The stimulus intensity that evoked a fEPSP of 50-60% of the maximal response was chosen to acquire the baseline response (15 min) for all experiments. For short term plasticity experiments, a fEPSP of  $\sim$ 50% of the maximum was obtained and two stimuli were delivered with a 120 ms inter-stimulus interval (ISI). Paired Pulse Ratio (PPR) was calculated as fEPSP2/fEPSP1 (3 sweeps average) right before and after BZY bath-perfusion. For all experiments, BZY was added at the final concentration to the perfusion aCSF. Preliminary experiments showed that at the concentration of 30  $\mu$ M BZY was effective in decreasing the PLCx-NAcc fEPSP by about 50% after 30 min of exposure, whereas at the concentration of 100  $\mu$ M fEPSP was virtually abolished. Thus, whereas in the acute perfusion experiments the exposure time was 10 minutes (Fig. 3), in the long term plasticity study the exposure time to 100  $\mu$ M BZY was reduced to 8 min (Fig. 5).

### **Data analysis and statistics**

Data were analyzed using IBM SPSS 20 statistical software. *In vivo* data were analyzed using a 4-way mixed ANOVA with between-subject factors drug experience (drug naïve vs. drug-experienced) and dose (3 levels) and with repeated measures on the factors lever (active vs. inactive) and session (14 levels). Electrophysiological data were analyzed using ANOVAs with repeated measures on the factor time. Data from paired pulse experiments were analyzed using paired samples t-test.

## RESULTS

### BZY self-administration

Figure 1 illustrates the reinforcing effects of BZY on lever pressing as a function of session and drug experience. The acquisition of BZY self-administration was greatly facilitated in drug-experienced rats relative to drug naïve rats. A 4-way ANOVA indicated a significant main effect of lever [ $F_{1,17}=30.008$ ;  $p<0.001$ ], session [ $F_{13,221}=8.646$ ;  $p=0.001$ ], and drug experience [ $F_{1,21}=7.721$ ;  $p=0.013$ ]. There were also lever\*session [ $F_{13,221}=10.511$ ;  $p<0.001$ ] and lever\*drug experience [ $F_{1,21}=5.629$ ;  $p=0.03$ ] interactions. Furthermore, a 3-way ANOVA of the number of infusions yielded a main effect of session [ $F_{13,221}=2.672$ ;  $p=0.014$ ] and of drug experience [ $F_{1,17}=14.564$ ;  $p=0.001$ ], as well as session\*drug experience [ $F_{13,221}=3.183$ ;  $p=0.004$ ] and session\*training dose [ $F_{26,221}=2.119$ ;  $p=0.016$ ] interactions (data not shown).

### Electrophysiological recordings

Bath applications of BZY at concentrations of 30  $\mu$ M for 30 min or 100  $\mu$ M BZY for 10 min decreased PLCx-NAcc fEPSP amplitude by  $36.1\pm6.8\%$  (from  $0.77\pm0.06$  mV to  $0.51\pm0.09$  mV,  $n=7$ ) and  $64.8\pm5.7\%$  (from  $0.75\pm0.07$  mV to  $0.27\pm0.05$  mV,  $n=7$ ), respectively (Fig. 2 and 3). Repeated measures ANOVA run separately for 30  $\mu$ M and 100  $\mu$ M experimental groups on the fEPSP amplitude from the time period 15-45 showed a significant effect of time for both the 30  $\mu$ M [ $F_{60,360}=7.954$ ;  $p<0.001$ ] and the 100  $\mu$ M concentration [ $F_{60,360}=18.774$ ;  $p<0.001$ ]. Furthermore, 2-way repeated measures mixed ANOVA conducted on data from the time period 15-25 min yielded significant main effects of time [ $F_{20,240}=9.763$ ;  $p<0.001$ ], concentration [ $F_{1,12}=25.583$ ;  $p<0.001$ ], and a time\*concentration interaction [ $F_{20,240}=5.253$ ;

$p < 0.001$ ]. To exclude that this severe depression in synaptic transmission could be due to non-specific toxic effects of BZY, pulses of higher current intensity were delivered at the end of each experiment. Under these experimental conditions the depressed fEPSPs recovered to the baseline amplitude values (data not shown).

We also performed short-term plasticity experiments by measuring PPRs to assess if BZY-mediated effects are due to alterations of neurotransmitter release probability. In control conditions, the second synaptic response (fEPSP2) was smaller than the first (fEPSP1) (Fig. 2d), a phenomenon termed paired pulse depression. Benzydamine perfusion (30  $\mu$ M) further decreased PPR (from  $0.85 \pm 0.05$  baseline to  $0.59 \pm 0.03$  after 30 min perfusion;  $n=7$ ;  $t_6=4.978$ ;  $p=0.003$ ), suggesting a presynaptic mechanism of drug action (Creager et al., 1980; Wu and Saggau, 1994). Due to the deep depression of the fEPSP induced by 100  $\mu$ M BZY (Fig. 3), PPR data collected from these experiments were not included in the analyses.

The fEPSP depression induced by BZY was significantly enhanced in drug-experienced animals compared to naïve rats (Fig. 4). Two-way mixed ANOVA for repeated measures unveiled a significant main effect of experience [ $F_{1,21}=9.237$ ;  $p=0.006$ ] and experience\*time interaction [ $F_{59,1239}=3.573$ ;  $p=0.01$ ], indicating sensitization of PLCx-NAcc synapses to acute effects of BZY perfusion, in drug-experienced rats.

As shown in figure 5a, bath application of 100  $\mu$ M BZY for 8 min induced long term depression of fEPSP amplitude with a peak of  $-60.1 \pm 5.0\%$  at min 25-35 (from  $0.55 \pm 0.04$  mV to  $0.22 \pm 0.03$  mV;  $n=9$ ;  $t_8=9.984$ ;  $p < 0.001$ ), indicating that BZY can cause long-term changes of PLCx-NAcc synaptic connectivity. Longer time of BZY perfusion often resulted in the complete suppression of the fEPSP (Fig. 3). The fEPSP amplitude did not fully recover during

the wash out (from  $0.55 \pm 0.04$  mV baseline to  $0.36 \pm 0.03$  mV at time 70-80 min;  $t_8=6.347$ ;  $p<0.001$ ) and was still reduced ( $-33.4 \pm 4.5\%$ ) at the end of recordings. This stable LTD-like response was consistently reduced by the co-application of the CB1 receptor antagonist AM251 ( $F_{1,19}=18.635$ ;  $p<0.001$ ; Fig. 5A). In the presence of AM251  $2 \mu\text{M}$ , BZY-induced fEPSP depression was smaller than in the BZY group both at the depression peak ( $-35.8 \pm 5.7\%$  vs.  $-60.1 \pm 5.0\%$ ;  $n=12$ ;  $t_{19}=3.071$ ;  $p=0.006$ ) and at the end of recordings ( $-13.9 \pm 3.5\%$  vs.  $-33.4 \pm 4.5\%$ ;  $t_{19}=3.420$ ;  $p=0.003$ ). Repeated measures mixed ANOVA on PPR data showed a significant time point\*treatment interaction [ $F_{2,40}=5.548$ ;  $p=0.007$ ]. The reduction of PPR by BZY (from  $0.85 \pm 0.03$  baseline to  $0.73 \pm 0.04$  in the BZY group;  $t_8=2.664$ ;  $p=0.012$ ) was fully counteracted by AM251 co-perfusion (from  $0.86 \pm 0.05$  baseline to  $0.93 \pm 0.07$  in the BZY+AM251 group;  $t_{11}=1.577$ ;  $p=0.143$ ; Fig 5B). AM251 did not affect PPR *per se* ( $0.93 \pm 0.06$ ,  $p=0.175$  vs baseline PPR, data not shown). These results suggest that BZY requires the presence of available CB1 receptors to long-term and fully inhibit excitatory synaptic transmission at PLCx-NAcc-Core synapses.

## DISCUSSION

We report here three major findings. First, we found that BZY, a non-steroidal anti-inflammatory drug, is self-administered intravenously by rats and that the reinforcing effects of BZY are greatly enhanced by a history of heroin and cocaine self-administration. Second, we found that BZY induces LTD-like plasticity at PLCx-NAcc synapses. Third, we found that the latter effect was significantly reduced by the CB1 receptor antagonist AM251, suggesting a cannabinoidergic mechanism of BZY action.

The finding that BZY is reinforcing in the rat sheds a light on the abuse liability of medications containing BZY. Abuse of BZY is particularly well documented in Brazil (Nappo et al., 1993; Opaleye et al., 2009, 2011) but there have also been case reports from other countries, including Italy (Malavasi et al., 2012; Settini et al., 2012), Poland (Anand et al., 2007) and Turkey (Balaban et al., 2013). Most cases of voluntary systemic overdose of BZY concerned individuals with a history of substance abuse. Our study demonstrates that BZY acts as a positive reinforcer in both naïve rats and in drug-experienced rats. Notably, pre-exposure to cocaine and heroin facilitated the acquisition of BZY self-administration at lower unit doses, which were not effective in naïve rats.

Our study also reveals short and long-term changes of the excitatory synaptic transmission in the Prelimbic Cortex to Nucleus Accumbens circuitry elicited by BZY. In particular, acute BZY reduced PLCx-NAcc glutamatergic neurotransmission via a presynaptic mechanism. Similarly to the *in vivo* drug effects, BZY-induced LTD was potentiated in rats that had previously self-administered cocaine and heroin. Interestingly, previous studies have shown that cocaine and d-amphetamine can also depressed excitatory neurotransmission at the same synapse via dopamine receptors type 1 (Nicola et al, 1996). Yet, BZY-mediated glutamatergic effects do not appear to be dependent on dopaminergic mechanisms since the selective D1 antagonist SCH23390 did not affect BZY-induced reduction of fEPSP (data not shown).

The LTD of glutamatergic transmission induced by BZY seems to rely at least partially on the presence of available CB1 receptors on the glutamatergic presynaptic terminals. Indeed, the absence of BZY-induced changes in the paired pulse ratio in slices perfused with the CB1R antagonist AM251, suggested that CB1Rs took part in the induction of this

pharmacological form of long term synaptic plasticity. The mechanism by which BZY interacts with CB1 receptors remains unclear. BZY might act either as a direct agonist onto CB1 receptors or by stimulating endocannabinoid synthesis and release, both of which in turn reduce glutamate release from the cortical terminal (Domenici et al., 2006). Notably, WIN 55,212,2 is able to induce persistent and long lasting fEPSP amplitude depression in the NAcc (Robbe et al., 2002). Nevertheless, the fact that AM251 dampens but not abolishes BZY-induced LTD response does not allow us to exclude other components of this LTD that may rely on other neurotransmitters or neuromodulators. As far as long term plasticity is concerned, a similar outcome was obtained for amphetamine which has been found to induce a CB1-dependent LTD in the rat amygdala (Huang et al., 2003). It is intriguing to compare the similar synaptic actions of amphetamine and BZY with their stimulant properties. As reported in many drug forums, BZY behaves as a stimulant other than a hallucinogen. While there is no evidence for animal self-administration of primary hallucinogenic substances like LSD, mescaline (Deneau et al., 1969) and DOM (Yanagita, 1986), several reports describe animal self-administration of amphetamine derivatives which have both hallucinogenic and psychostimulant effects like MDMA and similar molecules (Fantegrossi et al., 2002; Lamb and Griffiths, 1987; Schenk et al., 2003, 2007; Sannerud et al., 1996). Thus, drugs acting selectively on serotonin receptors do not seem to be reinforcing, whereas substances that are active on multiple monoamine systems sustain operant behavior in animals. Taken together, these observations strongly suggest that BZY has a mixed effect on the CNS, with the probable involvement of several neurotransmitter systems.



The effects of CB1R on BZY-induced synaptic plasticity provide a potential mechanism for the abuse potential of BZY. Interestingly, the chemical structure of BZY shares some features (like the presence of a benzoyl indole) with several CB1 synthetic agonists (i.e. JWH series compounds), which were shown to possess hallucinogenic properties as well (Forrester, 2012; Harris and Brown, 2013).

In conclusion, these data provide the first empirical evidence of BZY abuse liability and its effect on central nervous system, suggesting that it can interact with cannabinoidergic signaling in brain structures critically involved in drug reward. Our findings suggest that individuals with a history of substance abuse might be more prone to develop BZY abuse not only as a consequence of a general predisposition to experiment with psychoactive substances, but also because of a specific pharmacological cross-sensitization to its rewarding effects. This could be of particular relevance for the management of detention facilities or rehabilitation clinics, considering that an addict might consume BZY (or similar substances) as a substitute drug without even being noticed by supervisors, thus unpredictably compromising health conditions along with therapy efficacy. Further research is needed in order to better dissect BZY effects on glutamatergic PLCx-NAcc transmission, understand its abuse potential, and possibly redefine the toxicological profile of this drug.

## **Funding and Disclosure**

### **Author contribution**

The study was designed by AB, SM, MM, RA, EM, and ES. RA, MM, and ES conducted the experiments under the supervision of AB and SM. Data analysis was conducted by RA, AB, and SM. RA and AB drafted the manuscript, which was critically reviewed by SM. All authors approved final version for publication.

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## Figure Legends

**Figure 1.** Lever pressing behavior during 3 hours daily sessions of BZY self-administration. FR is scaled throughout the training as shown in figure. Drug experienced animals readily acquire the self-administration behavior for all dosages, while naïve animals do so only for higher dosages. Values are displayed as means  $\pm$  SEM.

**Figure 2.** Effect of BZY perfusion on PLCx-NAcc glutamatergic fEPSP. **a)** Mean normalized fEPSP amplitude during perfusion of BZY 30  $\mu$ M (n=7 slices/3 animals, 30 min). **b)** Schematic of electrode placement with mediolateral coordinates from Bregma. **c)** Representative time-course of an experiment for BZY 30  $\mu$ M perfusion with sample traces from different time points. Traces were obtained averaging 3-4 sweeps. **d)** Paired Pulse Ratio (PPR) values measured right before and right after the 30 minutes perfusion of BZY 30  $\mu$ M. BZY induced a significant reduction in PPR at PLCx-NAcc synapses. Representative traces are displayed

above the histogram. \* indicates significantly different ( $p < 0.05$ ) from preBZY PPR. Values are displayed as means  $\pm$  SEM.

**Figure 3.** Effect of BZY perfusion on PLCx-NAcc glutamatergic fEPSP. **a)** Mean normalized fEPSP amplitude during perfusion of BZY 100  $\mu$ M ( $n=7$  slices/3 animals, 10 min). **b)** Schematic of electrode placement with mediolateral coordinates from Bregma. **c)** Representative time-course of an experiment for BZY 100  $\mu$ M perfusion with sample traces from different time points. Traces were obtained averaging 3-4 sweeps. **d)** Paired Pulse Ratio (PPR) values measured right before and right after the 30 minutes perfusion of BZY 30  $\mu$ M. Representative traces are displayed above the histogram. Values are displayed as means  $\pm$  SEM.

**Figure 4.** fEPSP depression induced by 30  $\mu$ M BZY is enhanced in drug experienced rats' brain slices. **a)** BZY-induced fEPSP depression in brain slices from naïve ( $n=7$  slices/4 animals) and drug experienced rats ( $n=10/5$ ). **b)** Representative traces from experiments on brain slices collected from naïve or drug experienced rats. Traces were obtained averaging 3-4 sweeps at baseline and the end of the 30 min BZY perfusion. Values are displayed as means  $\pm$  SEM.

**Figure 5.** BZY is able to induce stable LTD at the PLCx-NAcc synapses. **a)** Normalized fEPSP amplitude in response to 100  $\mu$ M BZY perfusion for 8 min ( $n=10$  slices/6 animals). AM251 2  $\mu$ M co-perfusion significantly reduces the BZY-induced LTD ( $n=12/8$ ). **b)** Paired Pulse Ratio values measured at different time points (as depicted by numbers) show that BZY-mediated alteration of glutamate release were significantly inhibited by the CB1 antagonist AM251. \* indicates significantly different ( $p < 0.05$ ) from BZY group, same time point (2); # indicates significantly different ( $p < 0.05$ ) from BZY baseline (1). Values are displayed as means  $\pm$  SEM.





Figure 1

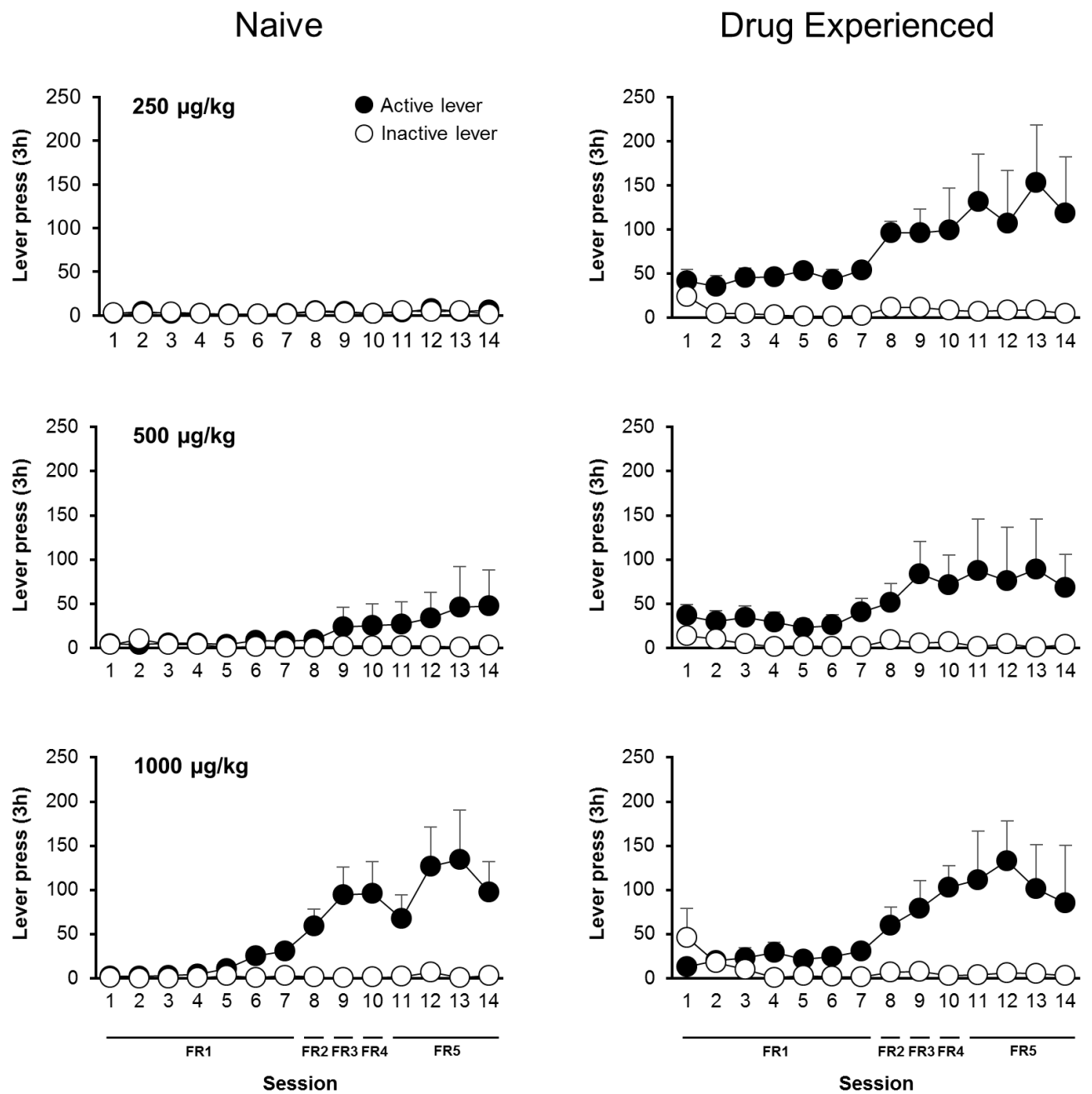


Figure 2

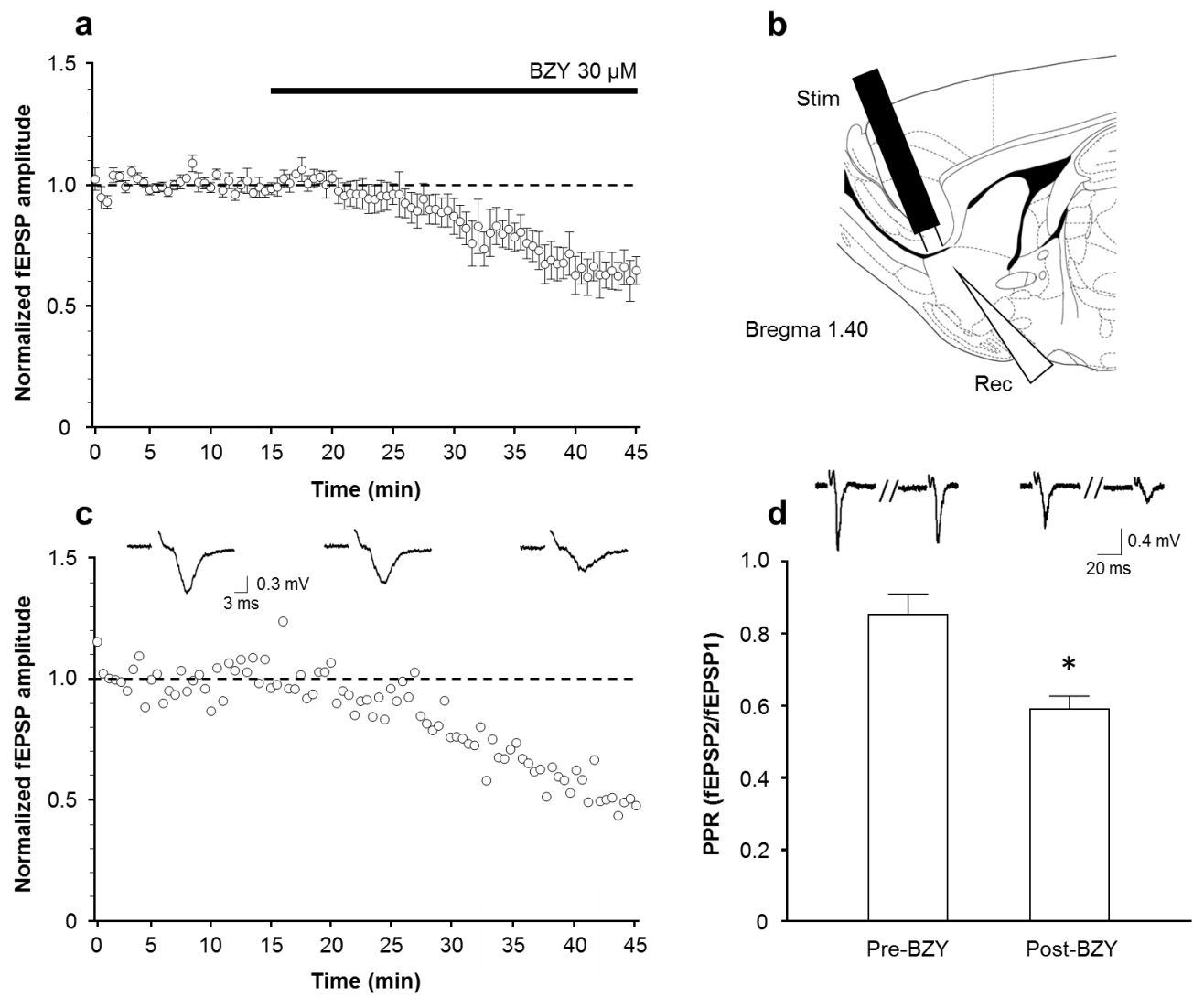


Figure 3

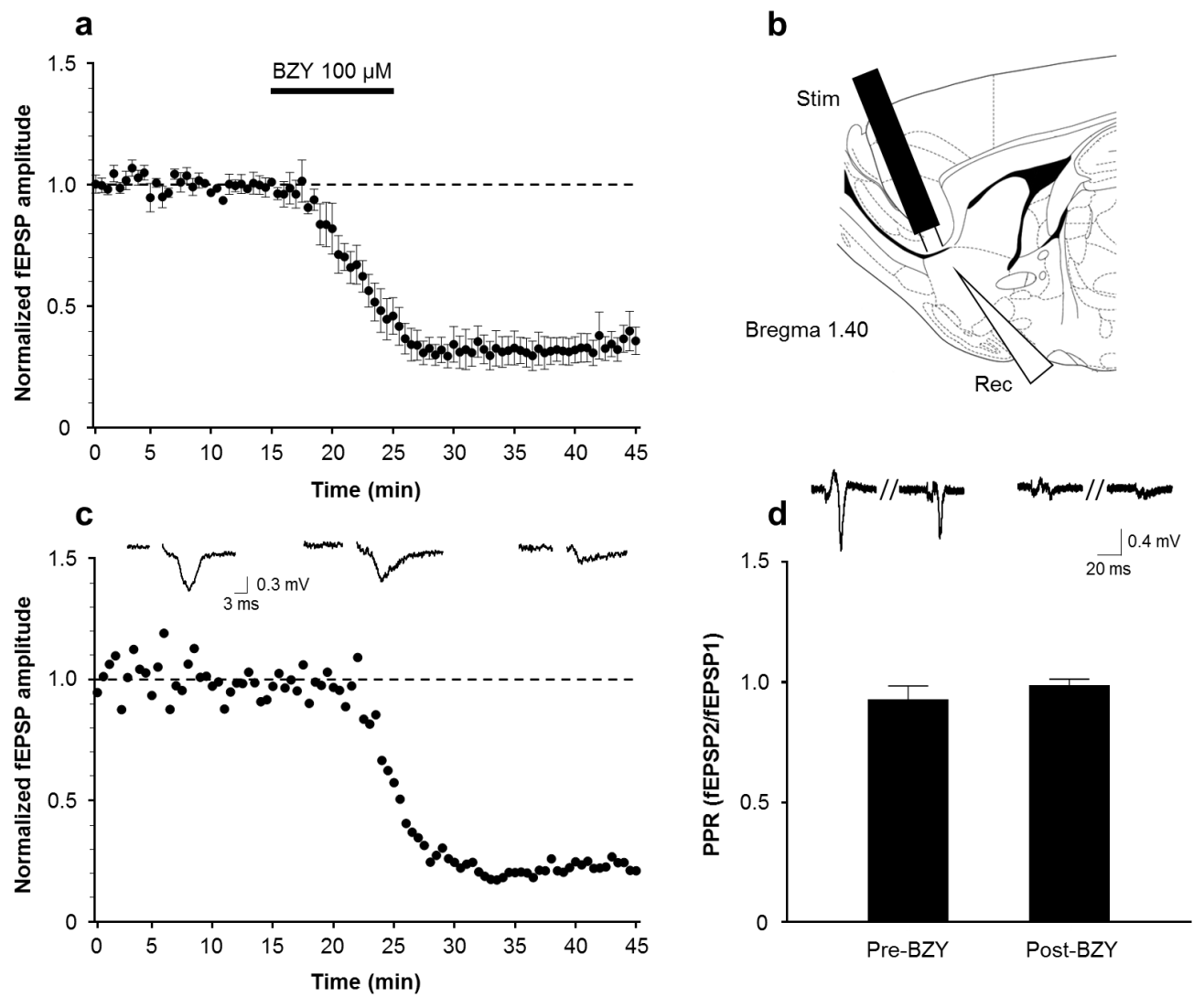


Figure 4

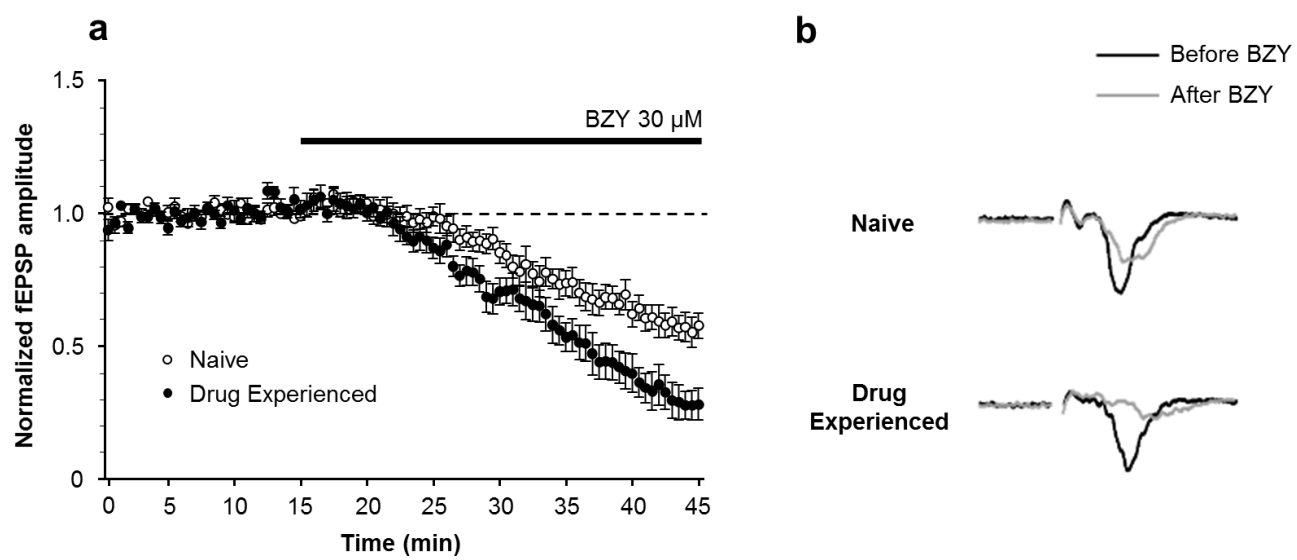


Figure 5

